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Ultra-trace graphene oxide in a water environment triggers Parkinson's disease-like symptoms and metabolic disturbance in zebrafish larvae

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ABSTRACT

Over the past decade, the safety of nanomaterials has attracted attention due to their rapid development. The relevant health threat of these materials remains largely unknown, particularly at environmentally or biologically relevant ultra-trace concentrations. To address this, we first found that graphene oxide (GO, a carbon nanomaterial that receives extensive attention across various disciplines) at concentrations of 0.01 μg/L–1 μg/L induced Parkinson's disease-like symptoms in zebrafish larvae. In this model, zebrafish showed a loss of more than 90% of dopamine neurons, a 69–522% increase in Lewy bodies (α-synuclein and ubiquitin) and significantly disturbed locomotive activity. Moreover, it was also shown that GO was able to translocate from the water environment to the brain and localize to the nucleus of the diencephalon, thereby inducing structural and morphological damage in the mitochondria. Cell apoptosis and senescence were triggered via oxidative stress, as shown by the upregulation of caspase 8 and β-galactosidase. Using metabolomics, we found that the upregulation of amino acid and some fatty acids (e.g. dodecanoic acid, hexadecanoic acid, octadecenoic acid, nonanoic acid, arachidonic acid, eicosanoic acid, propanoic acid and benzenedicarboxylic acid) metabolism and the downregulation of some other fatty acids (e.g. butanoic acid, phthalic acid and docosenoic acid) are linked to these Parkinson's disease-like symptoms. These findings broaden our understanding of nanomaterial safety at ultra-trace concentrations.

1. Introduction

Due to the extraordinary properties of nanomaterials, they have been widely produced and utilized in various fields. With the rapid development and application of nanomaterials, their safety has attracted considerable attention [1,2]. Currently, graphene-family nanomaterials (GFNs) are in commercial production [3]. With the rapid increases of GFNs in production and application [4], GFNs will likely release into the environment at significant levels. For example, grapheme oxide-polymer (GO-polymer) nanocomposites could release GO particles during their life cycle [5]. Notably, considerable GFN release could occur during environmental applications such as adsorbents for drinking water and wastewater treatments [6,7], catalysts for aqueous organic pollutant degradation [8], and coating materials for filtration [9]. In addition, GO could be introduced to the environment during the waste disposal of GO-containing products. Understanding the adverse effects of nanomaterials is critical to their optimization and administration [10]. Recently, some data concerning material safety have been reported. For example, GO adversely affected cell function by decreasing cell viability and increasing reactive oxygen species (ROS) generation, thereby activating inflammatory and apoptotic pathways. Noticeably, the responses of cellular levels or the *in vitro* experimental conditions did not directly reflect the health consequences of the nanomaterials, including the occurrence of diseases [10,11]. Moreover, it has been reported that biological responses linked to nanomaterial concentrations [12,13]. Unfortunately, the safety of nanomaterials, particularly in the evaluation of disease risks, remains largely unclear at environmentally or biologically

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relevant ultra-trace concentrations.

According to estimates derived from mathematical models, the concentrations of engineered nanomaterials in the effluent of sewage treatment plants and in surface water are at parts per billion (ppb) and parts per trillion (ppt) levels [14,15]. Therefore, experiments that make use of nanomaterials at concentrations of parts per million (ppm) to evaluate environmental risks make little sense to nano safety, making it difficult to extrapolate the results of these studies to human or ecological health. Nanomaterials have also been directly employed in various fields, where the initial concentrations used can be at the high ppm level [16,17]. Nevertheless, according to pharmacokinetics, most nanomaterials would be gradually cleared via the processes of renal and fecal excretion, which would reduce accumulated concentrations in the body [18]. Therefore, the concentrations of exposure for determining environmentally or biologically safe levels should be emphasized at either the ppb or ppt levels. Furthermore, there are few reports concerning nanomaterial exposures at ultra-trace concentrations. To address this, we tested 0.01 μg/L–1 μg/L of graphene oxide (GO, a carbon nanomaterial that has received extensive attention in various applications).

Parkinson's disease (PD) is the world's second most common neurodegenerative disease. Recently, there have been increasing reports indicating that some nanoparticles can result in neurodegeneration. TiO_2 and Ag nanoparticles that enter into primary neuronal cells stimulate ROS in brain microglia and damage neurons *in vitro* due to caspase activation-mediated signaling [19–21]. SiO_2 nanoparticles resulted in the deterioration of cognitive behavior and locomotive activity in zebrafish and posed a negative impact on the striatum and dopaminergic (DA) neurons [22,23]. Carbon nanotubes also could cross the cell barrier, thereby inducing cognitive deficits and decreasing locomotor activity while increasing levels of oxidative stress and apoptosis in mouse brains [24,25]. GO is unlike zero-dimension nanoparticles and one-dimension carbon nanotubes as it has specific toxic properties that result from its single layer of flexible, two-dimensional shape [1,2,26]. Presently, whether GO can be absorbed from the environment into the brain, how GO is absorbed into the brain and the role of GO as a potential neurotoxin that can promote PD-like symptoms are unclear.

Common mechanisms of PD-like symptoms include mitochondrial dysfunction, increases in oxidative stress, protein misfolding aggregation, and gene changes in fish, mouse and human. In contrast to the above biochemical parameters, metabolites serve as direct signatures of biochemical activity and are readily correlated with cellular biochemistry and biological phenomena [27,28]. Recently, global and untargeted metabolomics technology has been considered a new strategy to reveal the mechanisms of toxicity or disease [29,30]. However, metabolic disturbances induced by nanomaterials have been ignored. Herein, it is hypothetical that investigating metabolic disturbance may provide new insights into understanding the mechanisms of PD-like symptoms that are induced by nanomaterials.

When considered together, it is necessary to determine whether GO might induce PD disease-like symptoms at environmental or biological concentrations, and its role in the pathogenesis thereof. Therefore, this work investigated the PD-like symptoms in the model organism zebrafish that were induced by ultra-trace concentrations of GO, including the loss of DA neurons, the promotion of Lewy bodies (α-synuclein and ubiquitin), and disturbances in locomotive activity. Moreover, the translocation of GO from the environment to brain cells and associated mitochondrial damage were studied. Subsequently, the rate of apoptosis and changes in the levels of senescence-related proteins and ROS were explored. Finally, metabolic disturbances linking to PD-like symptoms were analyzed using a metabolomics strategy.

2. Materials and methods

2.1. Fish maintenance

Adult zebrafish (AB strain, 6 months) were maintained at 28 °C in a recirculating aquaculture system that was equipped with a mechanical filter and an aquarium heater. Zebrafish were kept under a 14 h/10 h light/dark cycle. Fish were fed twice daily using brine shrimp. Larval zebrafish were obtained from adult fish through natural mating. Larval zebrafish were cultured in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.4) at 28 °C in a climate cabinet (SPX-300I-C, BOXUN, China).

2.2. Nanomaterial characteristics and biological exposure

GO was purchased from XFNANO (XF002-1, Nanjing, China). Atomic force microscopy (AFM) and field-emission transmission electron microscopy (TEM) studies were conducted with a Nanoscope 4 (Veeco, USA) and a JEM-2010 FEF (JEOL, Japan), respectively, to study nanomaterial morphology. X-ray photoelectron spectroscopy (XPS) measurements were conducted using an Axis Ultra XPS system (Kratos, Japan) with a monochromatic Al Kα X-ray source (1486.6 eV) to measure the nanomaterial surface chemical groups. The spectra were analyzed using Casa-XPS V2.3.13 software, and the peaks were deconvoluted using Gaussian components after performing a Shirley background subtraction. The reactions between free radicals and nanomaterials were detected using an electron paramagnetic resonance (EPR) spectrometer (Magnettech MiniScope 400, Germany) with MiniScope Control software. EPR was operated at a microwave frequency of 9.4 GHz and a magnetic field modulation frequency of 100 kHz at 296 K. 2,6,6-tetramethyl-1-piperidinyloxy (TMPO) was employed as an unpaired electron probe. The zeta potentials were obtained by performing dynamic light scattering using a ZetaPALS instrument that was equipped with a 30 mW 635 nm laser (BI-200SM, Brookhaven, USA). GO was dissolved in E3 embryo culture medium. Hydrodynamic diameter (Hd) of GO in E3 embryo culture medium was detected to evaluate agglomeration behavior of GO in the aquaculture system at 0 h and 24 h by Zetasizer Nano ZS90 (Malvern, UK). Embryo cultivation and analysis were conducted according to OECD standardized testing guidelines-Fish Embryo Toxicity (FET) Test, 2006. The media was changed daily, according to the ISO 7346-2:1996 guideline. Larval zebrafish at 72 h post fertilization (hpf) were treated with 200 μL E3 embryo culture medium (control), 0.01 μg/L, 0.1 μg/L and 1 μg/L GO for 24 h in 96-well plates at 28 °C in a climate cabinet with a 14 h/10 h light/dark cycle.Upon exposure, the larval fish were rinsed by E3 embryo culture medium three times and then mortality, malformation, immunohistochemistry, western blotting, senescence associated β-galactosidase, behavior analysis, reactive oxygen species, transmission electron microscopy, raman spectra and metabolic alterations were analyzed. In addition, 1-methyl-4-phenylpyridinium (MPP^+), a typical toxin inducing Parkinson's disease, was used as a positive control. Larval zebrafish at 72 hpf were treated with 200 μL E3 embryo culture medium with MPP^+ with typical concentrations (2.98–74.5 mg/L) that induced obvious neurotoxicity. Reactions were performed for 24 h in 96-well plates at 28 °C in a climate cabinet with a 14 h/10 h light/dark cycle. Then number of movements of zebrafish during 3 min was recorded.

2.3. Mortality, malformation and immunohistochemistry

The mortality and malformation of larval zebrafish were recorded at 120 hpf via light microscopy (Olympus ZL 61, Olympus,